REMARKS

The foregoing amendments and these remarks are filed in response to the Final Office Action dated September 29, 2010 (the "Office Action"). This response is timely filed. The Commissioner is hereby authorized to charge the required fee for a Request for Continued Examination, as well as any deficiency in fees, to Deposit Account No. 50-0951.

At the time of the Office Action, claims 159-181 were pending, with claims 182-185 being withdrawn from consideration by the Examiner as being directed to a non-elected invention. Claims 159-181 were rejected under 35 U.S.C. §103. All of the rejections and responses thereto are set out fully below.

I. Rejection under 35 U.S.C. §103(a)

Claims 159-168, 171-176, and 178-180 were rejected under 35 U.S.C. §103(a), as being unpatentable over U.S. Patent Publication No. 2002/0197611 to Chagovetz et al. ("Chagovetz"), Solinas et al., "Duplex Scorpion Primers in SNP Analysis and FRET Applications," Nucleic Acids Research, vol. 29, no. 20, pp. 1-9, October 15, 2001 ("Solinas") and U.S. Patent No. 6,117,635 to Nazarenko (2000) ("Nazarenko (2000)"). Claims 159-168, 171-176, and 178-180 are rejected under 35 U.S.C. 103(a) as being unpatentable over Chagovetz, WO 1998/13524 to Sato et al. ("Sato") and Nazarenko (2000). Lastly, claim 181 is rejected under 35 U.S.C. 103(a) as being unpatentable over (1) Chagovetz, Solinas and Nazarenko (2000) as applied to claim 159 above, or (2) Chagovetz, Sato and Nazarenko (2000) as applied to claim 159 above; and further in view of Webb et al., Accession No. M60048, 1993 ("Webb") and Buck et al., "Design Strategies and Performance of Custom DNA Sequencing Primers," BioTechniques 27:528-536, September 1999 ("Buck"). Applicant respectfully submits that claim 159 is patentable over the cited art.

Prior to addressing the prior art, a brief review of an aspect of Applicant's invention is appropriate. Applicant notes that it is difficult to avoid or eliminate primer-dimer formation. A few solutions addressing primer-dimer formation have been reported, such as the S. Das et al., Biotechnology Techniques 13: 643–646, 1999 and Brownie, J. et al., The elimination of primer-

dimer accumulation in PCR, Nucleic Acids Research, 1997, Vol. 25, No. 16 references. Notably, in the absence of a good solution to primer-dimer formation, an additional probe is used for specificity of target detection. The features of claims 159 recite a new and non-obvious technique for eliminating primer-dimer formation in nucleic acid amplification, as well as for target detection. Claim 159 relates to formation of primer-dimer when a label is placed on each of two primers of a nucleic acid amplification, and is placed on a nucleotide that is separated by less than two nucleotides from the 3' end of the primers. Further, when the labels are placed on each of two primers on a nucleotide that is separated by two to four nucleotides from the 3' end of the primers, there would not be any primer-dimer formation. Such specific selection of placement of labels on primers addresses the problem of primer-dimer formation, a major problem of the field and for which there was no good solution prior to the development of the features of claim 159. Selection of such labeled primer pairs allows annealing of primers at an optimum lower annealing temperature range and for a longer duration of time. This technique improves the yield of the amplification product, resulting in higher sensitivity of target detection. Moreover, under this approach, there is amplification when the labeled primer pair is so designed that the nucleotides carrying label would remain separated by less than four nucleotides on the amplification product. In addition, under this approach, amplification is a few folds less even when two nucleotides carrying the labels are separated by four intervening nucleotide pairs on amplification product. Moreover, when the labels are separated on amplification product by ten intervening nucleotide pairs, the yield of the amplification product is good (equal to the yield obtained with unlabeled primers). The crowding of the labels is quite significant. It interferes with the binding of the polymerase to the 3' end of the primer and formation of primer-template-polymerase complex before extension. The labels can be fluorophore, fluorescent dye, hapten, nano-particle or any other entities. No prior art teaches these features.

The best way to amplify a target sequence without any primer-dimer formation is to label both primers with a label on a nucleotide, which is separated by two to four nucleotides from 3' end, and to have at least four nucleotide pairs separate the two nucleotides to which two labels are attached in the amplification product (i.e., two dyes are at a distance of at least six

nucleotides). Further, the selection of the label positions are not based on FRET, where FRET is only a preferred mode of signal generation. It is based on the efficiency of amplification at different distances between two dyes on amplification product, and avoiding of primer-dimer formation. The features of claim 159 teach toward avoiding and eliminating primer-dimer formation in a manner that is notably different than the prior art. Turning to the prior art, the selection of placements of labels taught by Chagovetz is significantly different from that which is recited by claim 159.

Paragraph 8 of the Office Action refers to Figures 7 and 8 of Chagovetz and mention that the acceptor (A) and the donor (D) are at least 1 base away from the 3' end, adapted to inhibit signal from primer-dimer and eliminate non-specific signal generation. Such separation will not inhibit primer-dimer formation, and instead will increase primer-dimer formation and non-specific signal generation, page 7, lines 30–39. Chagovetz mentions that the donor acceptor labels on forward and reverse primers are separated by 1 nucleotide when they are incorporated into the amplification product, and there will be high signal intensity at relatively low concentration of amplicon. The reference also mentions that a FRET signal characteristic of the specific dye pair is expected to emerge above baseline at about 35 cycles of amplification for 10 initial mRNA copies.

An amplification of such type will not be amplified due to inhibition of the amplification caused by short separation between two dyes (see Fig 2, Table 1, and Fig 3, lines 2–8, page 2741, of Ahmad et al., Anal Bioanal Chem (2007) 387: 2737–2743 ("Ahmad"), an independent publication which shows that amplification will not occur). Hence, the examples of Chagovetz do not teach that which is recited by claim 159.

Moreover, primer-dimer will be formed under these conditions. As two primers of Chagovetz are labeled at the 3' end nucleotide, when the primers overlap each other by a few nucleotides, polymerase will not face any dye while extending labeled primers. As a result, primer-dimer product will be generated and amplified very well.

Turning now to Solinas, the signal from acceptor will be very weak at a distance of 3 nucleotides between the donor and acceptor. This product only indicates that the product formed

is a primer-dimer. Chagovetz has amplified a primer-dimer product, and has mistaken the same for a specific amplicon. It is very difficult to distinguish between a specific amplicon and a primer-dimer product because of their similar size, so whatever Chagovetz has tried to demonstrate in this example does not work. Under the conditions suggested by Chagovetz, amplification of the amplicon is inhibited and primer-dimer formation alone has taken place. The publication of Ahmad also supports this conclusion.

In contrast, in one aspect of the present invention, the nucleotides carrying the fluorophores should be separated by at least four nucleotide pairs on amplification product for a reasonable amplification. For separation less than that target amplification will get inhibited and amplification reaction will fail. Fig. 2, Table 1, and particularly Fig. 3 and lines 2–8 page 2741, of Ahmad establish this position. In contrast, according to Chagovetz's disclosure, amplification will work for any separation less than 24 nucleotides (less than 100 Angstrom), and the signal will be very intense when donor and acceptor are separated by minimal distance (paragraph 0022 of Chagovetz). Minimal separation is when two fluorophores are on consecutive nucleotides. In example [0035], Chagovetz has used separation of one nucleotide. Thus, the disclosure of Chagovetz for amplification of target where two fluorophores are maximally close does not work. Chagovetz does not teach that two fluorophores should be separated in amplicon by at least four intervening nucleotide pairs, which is a requirement for use of two primers each labeled with a donor or an acceptor in target amplification.

In addition, Chagovetz has applied annealing at high temperature and acquisition of fluorescent signal at 75°C to avoid primer-dimer formation and acquisition of signal from primer-dimer. The result of this example where there is amplification of an amplicon with separation of 1 nucleotide between fluorophores (as described above) establishes that application of annealing at high temperature and acquisition of fluorescent signal at 75°C will not be able to avoid primer-dimer formation and acquisition of signal from primer-dimer. Hence the teaching of Chagovetz does not disclose or suggest the features of claim 159. Differentiation between an amplicon and a primer-dimer by the teaching of Chagovetz is not possible. It should also be noted in this context that amplicons of small size and with two internal labels have very broad

melting temperature, and melting temperatures of amplicon and primer dimer are very close. Further, according to the formula for melting temperature (Tm = 75.1 + 11.7 Log [k+] + 0.41 (GC%) – 528 / L), Tms for Chagovetz's forward primer, reverse primer and amplicon are respectively 60.7, 62.9 and 75.5, meaning signal acquisition at 75 ºC cannot be used.

Further, selection of placement of fluorophores on the sixth or seventh nucleotide from 3' end of both primers, or one fluorophore on fifth nucleotide of one primer and the other fluorophore on the sixth or seventh nucleotide from 3' end of other primer, and the selection of a separation of less than ten nucleotide pairs between two primer ends on amplification product, as suggested by Chagovetz will generate primer-dimer. Application of high annealing temperature will not be able to eliminate primer-dimer formation, and acquisition of fluorescent signal at 75° C will not help in avoiding signal from primer-dimer. Hence, PCR amplifications will not work well as the signal from amplification product would be much less than that from primer-dimer making the target detection to fail because of low signal to noise ratio. In this context, it should be noted that a positive result in negative PCR control, i.e., the control amplification reaction in which no target sequence is added, will make the target detection assay to fail. Thus, the teachings of Chagovetz do not work at minimal range of separation between two fluorophores on amplification product (distance of less than six nucleotides between two dyes) and placement of labels at minimal distance from 3'end of primers; and at the same time it does not work at longer range of separation between two fluorophores on amplification product (placement of labels at maximal distance 5 – 7 nucleotides from 3'end of primers, and separation of ends of primers in amplification product by less than ten nucleotide pairs).

Chagovetz has selected position of labels on primers at the 1 – 7 nucleotide from the 3' end (Fig 1, page 3, lines 15-17, and page 4, lines 24–28). In a more preferred embodiment, the fluorescent label is placed on nucleotide 1 from the 3' end or very close to 3' end of each primer. Separation of the 3' ends of two primers on amplification product is separated by less than ten nucleotide pairs. These teachings emphasize that donor and acceptor moieties should be placed at minimal separation in double stranded amplification product for high intensity of FRET signal (page 4 lines 33 – 39). The above selections are not based on any technical finding. They are

based on different permutations and combinations of known FRET distance (1 – 24 nucleotides distance), and the fact that FRET signal is known to be more intense when FRET partners are in very close proximity. In stark contrast, Applicant invented a new solution to the primer-dimer problem, and teaches specific ways of using two labeled primers that will not inhibit target amplification but will inhibit primer-dimer formation. In sum, Applicant teaches that if two labels or fluorophores (on two primers) are placed very close on an amplification product at a separation of less than four nucleotide pairs, target amplification is inhibited, and primer-dimer formation takes place (please see example 7). This occurs when the amplicon and primer-dimer are of similar size, whereas Chagovetz has failed to do so. Applicant has studied the phenomena of inhibition of target amplification and formation of primer-dimer, and has arrived at a clever and judicious placement of label on each primer so that target amplification occurs without inhibition and no primer-dimer is formed. Applicant has used the range of distance at which there is amplification inhibition for inhibiting primer-dimer formation, and has selected the position of labels 2 to 4 nucleotides away from 3' end of each primer. This placement was not obvious to a person of ordinary skill at the time of the invention. The selection of the specific position of labeling of each primer is on nucleotide two to four bases away from 3' end. Further, the ends of two primers are separated by 0 – 21 nucleotide pairs on amplification product and not by 0 – less than 10 nucleotide pairs as suggested by Chagovetz. It is not an optimization but rather ability to see through when the things are not very clear cut or not very obvious, and a clever and careful choice of position of labeling on primers and separation of ends of primers, which is very intricate. Hence the findings of Applicant are highly non-obvious, and unexpected. To the contrary, the teaching of Chagovetz is of broad and obvious or speculative selection, which mostly does not work. Anybody interested in using two fluorophore labeled primers of Chagovetz for target amplification needs to reinvent the optimum selection of the position of the labels on the primers, optimum separation between ends of two primers, and optimum separation between two fluorophores on amplification product for successful target amplification. He also has to invent a solution to the primer-dimer problem.

Regarding Nazarenko (2000), there is no formation of a combination of two hairpin primers for generation of signal. No combination of two labels on two primers is formed and no signal from two labels on two primers is generated. There is no such information or teaching in Figure 23 and in description at column 11 lines 9 – 15 of the text of Nazarenko (2000) as asserted in the Office Action. It is not possible for this assertion to be correct for a number of reasons. First, hairpin primers are sluggish in opening. It is very difficult to design a good hairpin primer. Designing two hairpin primers within a very close proximity is even more difficult. Because of the sluggish opening of hairpin primer, amplification reaction becomes inefficient and amplification gets inhibited. Use of two hairpin primers will make the amplification reaction so sluggish and inefficient that there will not be any target amplification.

Regarding the Office Action assertion that the disclosure of Nazarenko (2000) for labeling of primer on an internal nucleotide, which is one nucleotide away from 3' end suggests the donor labels are internal by at least 2 bases from 3', one internal labeling cannot suggest another internal labeling. Types of internal labeling will depend on types of use. Same internal labeling cannot be used in two different methods. Internal labeling has been used in different ways and in different combination in prior art. For example Ju et al. (1995) Proc. Natl. Acad Sci. U.S.A., 92, 9347 – 9351 has used different dual labeled primers labeled internally in different positions in combination with additional labeling at 5' end for DNA sequencing. Nazarenko (2000) has used the same and other dual labeled primers (hairpin primer, donor-acceptor labeled linear primer, reverse primer-blocking oligonucleotide duplex primer) in target detection by PCR. Nazarenko (2000) has used internal labeling just for incorporation of a primer in order to directly detect a target. Applicant has used internal labeling on two separate primers in order to generate a signal from two labels on two labeled linear primers. The mode and purpose of use of internally labeled primers are different, and hence the requirements of placement of labels on primers would be different. Nazarenko (2000) uses internally labeled primer to incorporate the label into the amplification product and generate a signal through removal of quenching of the label, whereas Applicant uses two labels on two primers, incorporates two labels into the amplification product and generates a signal through energy transfer between two labels.

Similarly, the third oligonucleotide of claims 162, 166 and 167 is different from the third oligonucleotide of Nazarenko (2000). The function of the third oligonucleotide is different in claim 162 and claims 166 and 167. In claim 162 the labeled third oligonucleotide is a primer. A labeled first primer and an unlabeled second primer amplify a first segment of the target, and the third oligonucleotide primer amplifies a second segment of the first segment. The amplified second segment generates a signal. The third oligonucleotide of claims 166 and 167 is used for quenching of the fluorophore on the first oligonucleotide, which is a primer. The third oligonucleotide of Nazarenko (2000) is not an oligonucleotide, it is a third nucleotide sequence of the hairpin primer, which is a longer oligonucleotide made of four portions or four nucleotide sequences. Nazarenko (2000) has used the third oligonucleotide to form a duplex stem structure with the first oligonucleotide sequence of the hairpin primer. A donor is placed on third sequence and a quencher is placed on the first sequence. None of the first and third oligonucleotide sequences has priming ability; they are non-primer sequences. They have been used in a unique way to put a donor and a quencher very close in order to quench donor emission. The primer sequence of the hairpin primer is the fourth oligonucleotide sequence of the same. Thus, the use and purpose of the third oligonucleotide in Applicant's method and the use and purpose of the third oligonucleotide in Nazarenko (2000)'s method are different from each other. Similarly, nesting of an oligonucleotide in an amplification reaction can be done in many different ways depending upon the purpose. Mullis et al., who invented PCR, have disclosed nested PCR. Thereafter, nested PCR has been used in different ways in different methods. Applicant has applied nested PCR in its method, where the first and third primers are provided labeled separately with a donor and an acceptor, the third primer is nested between labeled first primer and unlabeled second primer. A signal is generated from the nested amplification product through interaction between the labels on first and third primer. Similarly Nazarenko (2000) use a hairpin primer to amplify a nested amplification product with two unlabeled primers. In Nazarenko (2000)'s method only the hairpin primer is labeled, and signal is generated from the hairpin primer alone.

Regarding Solinas, it appears that the Office Action has mistaken probe dimer for primer-dimer. A nucleic acid probe is an oligonucleotide, which is fully complementary to a target nucleic acid sequence. The function of a probe is to detect the target sequence in presence of other nucleic acid sequences. A probe is used in nucleic acid amplification to detect the amplified target sequence in presence of amplified non-specific or non-target products. Primer-dimer is a non-specific amplification product that is formed when two primers of nucleic acid amplification overlaps and polymerase extends two overlapped primers. It is an amplification product which is also an amplification artifact. A probe is used to detect a target amplification product in presence of non-specific amplification products. It is used to avoid acquisition of signal from non-specific amplification product including primer-dimer. Hence, it is natural that use of probe will help in avoiding acquisition of signal from non-specific product including primer-dimer. Use of a probe has its own associated problems of higher background, inhibition of amplification, and non-specific probe hybridization. Methods that do not use probes have the advantage of efficient target amplification, but also have the problem of acquiring signal from non-specific products, including primer-dimer, and hence there is a problem of specificity. There are special requirements for avoiding non-specific signal in non-probe based method. Hence probe based and non-probe based methods are two different types of methods, and cannot be compared. Further, inhibition of primer-dimer or non-specific amplification product by the duplex probe of scorpion does not arise, as the 3' end of the primer sequence of the scorpion is free, and it can form primer-dimer. Hybridization of the probe to amplified target will inhibit extension of the other primer by the polymerase. This inhibition will result reduction in yield of the amplicon and formation of more primer-dimer, and hence will reduce sensitivity of target detection. Therefore, a probe-based method cannot teach or suggest amplicon detection without probe.

The Office Action assertion that Applicant has modified labeled oligonucleotides of Chagovetz in combination with the disclosure of Solinas with reasonable expectation of success is not correct. Solinas' teaching regarding distance between fluorescent dyes is contrary to Applicant's findings. Solinas' teaching that donor and acceptor at three nucleotide distances

give weaker signals, and donor and acceptor at six nucleotides distances give maximum signals should not be mistaken as suggesting that donor and acceptor moieties can be placed at a separation of four nucleotide pairs to get an optimum signal though optimization. In Applicant's method, the minimum distance between two fluorophores for a reasonable signal is six nucleotides, not four nucleotides. In the method of Applicant, the nucleotides carrying donor and acceptor are separated by four nucleotide pairs. This may have led to the incorrect conclusion that Applicant has arrived at separation of four nucleotide pairs based on Solinas' teaching. According to Applicant's finding, it is not possible to place the donor and acceptor at less than six nucleotides distance as target amplification gets inhibited when donor and acceptor are placed on amplification product at less than six nucleotide distance. Even six nucleotide distance between donor and acceptor yield a fewfold less amplification product in comparison to the best result (see example 7 of Applicant's Specification).

Further, Becker et al. teaches the use of two unlabeled primers that are used for amplifying a target sequence, and a FRET labeled probe (labeled with a donor at one end and an acceptor at the other end, which are in energy transfer or FRET relation) is nested between the two primers. This was originally developed by Holland et al. Holland et al. has been referred to and admitted in present application (PCT application page 5 lines 21). The FRET labeled probe does not amplify any segment as it happens in nested nucleic acid amplification. Here the donor-acceptor dual labeled probe hybridizes to one strand of the amplification product and the probe is hydrolyzed by the 5' – 3' exonuclease activity of the polymerase resulting in separation of donor and acceptor and a signal from donor. This method of detection is in no way similar to that of the present invention, where signal generation occurs due to labeled primers. Holland and Becker have used labeled probe for signal generation. Since the word nested has been used, that does not mean that it can be related to any method using this term. In case of Becker et al. a FRET labeled probe has been nested between two amplification primers where the probe does not participate in amplifying target. Whereas in the method of Applicant, the third labeled (singly) oligonucleotide is a primer that participate in amplifying the target. Hence it is totally different from Becker et al.

Similarly, the third oligonucleotide primer of Applicant is different from third oligonucleotide primer of Chagovetz. The third oligonucleotide primer of Applicant amplifies one target in combination with other two primers, whereas the third oligonucleotide primer of Chagovetz amplifies a second target different from that is amplified by first and second primers. Hence they are different; it is the different contexts, which make similar things different.

Sato et al. teaches fluorophore labeled probes and not primers, and the process is a hybridization process and not an amplification process. The abstract clearly states that the invention provides detection with great accuracy and high sensitivity, particularly under the conditions where the probes may be abundant relative to the specimen in the sample. Further, the probes of Sato can never be used in nucleic acid amplification based target detection. The 3' ends of the probes are free and not blocked for polymerase extension. Hence polymerase will extend one of the probes and the other probe will generate non-specific product. The very purpose of using probe for specificity of target detection will be defeated as there cannot be any specific probe hybridization. Use of labeled probes will form more primer-dimer because increased concentration of oligonucleotides in the reaction. However, it can be applied to a target that has been amplified separately. Further, regarding the Office Action's assertion with respect to the teaching of FRET distance by Sato, separation distance between a donor and an acceptor is different for different donor-acceptor pairs and these distances are well documented. The optimum distance for optimum FRET between donor-acceptor of each pair is also known. One has to work within this limit. There is nothing to optimize about these distances. And donor-acceptor pairs with different optimum distances are available. For example, in case of donor FAM and acceptor ROX signal would be less in three-nucleotide distance between donor and acceptor, whereas signal would be very high in three-nucleotide distance between donor and acceptor when the donor is FAM and acceptor is JOE or Cy3 or FAM, and this is because of high energy transfer between them; whereas an acceptor like Rhodamine will not give any emission at such separation. Therefore, separation of three or less nucleotides (i.e., 7 Angstrom is less than lower limit of good FRET distance, which is 10 Angstrom) can be used for target detection with reasonably good sensitivity using specific FRET pairs. According to Applicant's disclosure it is

not possible. It is not possible, not because of FRET signal intensity, but because of failure of target amplification at separation of two nucleotides carrying donor and acceptor by less than 4 intervening nucleotides (i.e., distance of 6 nucleotides between two dyes) on amplification product. According to Applicant's disclosure, signal is reasonable at this distance as it is in the lowest bracket of his optimum distance. Thus, Sato does not give any clue as to what separation between two dyes should be used for sensitive target detection using two labeled primers. Similarly, it does not give any clue as to the distance from 3' end of the primers dyes should be placed so that good amplification can take place, and no primer-dimer formation will take place.

The separation of 4 – 20 or 25 nucleotide pairs, and particularly minimum separation of four nucleotide pairs between the donor and acceptor labeled nucleotides on amplification product in the invention of Applicant, is not related to FRET. It is not optimization of separation of donor and acceptor for optimum FRET signal. It is due to the fact that target amplification gets inhibited when donor and acceptor are separated by a distance of less than six nucleotides, which is equivalent to separation donor and acceptor labeled nucleotides by four nucleotide pairs. A distance of four nucleotides is equal to 10 Angstroms, which is a minimal distance for long distance FRET where a signal is generated from the acceptor. A distance of 20 nucleotides is equal to 70 Angstroms, which is a distance for a reasonably high signal for long distance FRET in case of certain donor-acceptor pairs. This is well known in the art and Sato has used this. A distance of four nucleotides in Sato is not the same as a distance of four nucleotide pair separation in Applicant's method. A distance of four nucleotide pair separation in Applicant's method is equal to distance of six nucleotides, i.e., 17 Angstroms. It is different from the distance of four nucleotides of Sato, and the basis of its selection is different from that of Sato. In Sato, the basis of selection is optimum FRET only, whereas in the method of Applicant, the most important basis of selection is not optimum FRET but rather optimum amplification by donor and acceptor labeled primers.

Further, Buck provides only a survey on primer construction and does not provide any motivation for quantification of PCR products.

Independent claim 159 is thus patentable over the cited prior art for the foregoing reasons. The dependent claims are also believed allowable because of their dependence upon an allowable base claim, and because of the further features recited therein.

II. Conclusion

Applicant has made every effort to present claims that distinguish over the prior art. All claims are believed to be in condition for allowance. Nevertheless, Applicant invites the Examiner to call the undersigned if it is believed that a telephonic interview would expedite the prosecution of the application to an allowance. In view of the foregoing remarks, Applicant respectfully requests reconsideration and prompt allowance of the pending claims.

Respectfully submitted,

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